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Establishment of an Experimental System for the Study of Tracheary Element Differentiation from Single Cells Isolated from the Mesophyll of *Zinnia elegans*

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ABSTRACT

Single cells were isolated mechanically from the mesophyll of adult plants and of seedlings of *Zinnia elegans* L. cv. Canary bird. When single cells isolated from the first leaves of seedlings were cultured in a liquid medium in the dark with rotation, they differentiated to tracheary elements with a reasonable degree of synchrony in the 24-hour period between days 2 and 3 after culture. The proportion of tracheary elements as a percentage of total cells reached nearly 30% 3 days after culture. Factors favoring cytodifferentiation were certain optimum levels of both α-naphthaleneacetic acid (0.1 milligram per liter) and benzyladenine (1 milligram per liter), a low concentration of ammonium chloride (0 to 1 millimolar), and an initial cell population density in the range 0.4 to 3.8×10^5 cells/ml. It was possible to follow analytically the sequence of cytodifferentiation in individual cells in this system.

The formation of tracheary elements is a dramatic case of cytodifferentiation in higher plants (12, 16) and may be regarded as a model system for the physiological (2, 17) and biochemical (6, 7) study of cytodifferentiation. Since most studies of tracheary element formation have been made using multicellular systems, it has been impossible to follow the sequence of cytodifferentiation to tracheary elements in individual cells. Similarly, the effect of cell to cell interaction makes the analysis of xylogenesis difficult. It is desirable, therefore, to establish an experimental system of tracheary element formation in a homogeneous single cell suspension culture. Torrey (14) reported that tracheary elements differentiated in a cell suspension culture of *Centaurea*. However, he found that the tracheary element formation in this system was of low frequency, variable, and asynchronous.

Kohlenbach (3) observed that isolated mesophyll cells of *Zinnia elegans* could form tracheary elements. We report here that establishment of a system for the study of tracheary element formation from single cells based on Kohlenbach's work.

MATERIALS AND METHODS

Plant Material. Seeds of *Z. elegans* L. cv. Canary bird were purchased from Takii Shubyo Co., Ltd., Kyoto, sterilized for 10 min in a solution of NaOCl (about 0.1%), rinsed repeatedly with sterile H_2O, and germinated in sterile moist Vermiculite in continuous light (4 klux, fluorescent) at 27 C for a week. The seedlings were grown in a controlled environment chamber with 12-h light periods (10 klux, fluorescent) at 28 C and 12-h dark periods at 25 C for a week in experiments using seedlings or 6 to 9 weeks in those using adult plants. The first leaves of seedlings and the youngest leaves of adult plants were used as plant material.

Isolation of Single Cells from the Mesophyll. Approximately 1.5 g of leaves were surface-sterilized for 10 min in a solution of NaOCl (about 0.05%), rinsed three times with sterile deionized H_2O, and cut into pieces (about 5 × 5 mm). The pieces were macerated with a pestle and mortar in the medium shown in Table I. By this treatment, the mesophyll cells were easily macerated to yield single cells, which were subjected to filtration through a 50-μm nylon screen and centrifugation at 150*g* for 1 min repeated once. The precipitated cells were collected and resuspended in 150 ml of the medium shown in Table I.

Culture Methods. Three-ml portions of the cell suspension were cultured in the dark in tubes (15 × 180 mm) at 27 C with rotation at 10 rpm in a rotating drum for 4 days in experiments using the seedlings and 8 days in those using the adult plants.

After a series of experiments using different salt formulations, we arrived at the following basal medium. The medium contained modified macroelements of Lin and Staba (4), microelements of Nitsch (9), organic growth factors of Nitsch and Nitsch (10), 0.1 mg/l NAA[1], 1 mg/l BA, 10 g/l sucrose, and 0.2 or 0.3 M mannitol. The composition of the medium is shown in Table I. The medium was adjusted to pH 5.5 before autoclaving.

Counting of the Number of Cells and Tracheary Elements. The number of cells and tracheary elements was counted by using a hemocytometer, Tatai type (Kayagaki Irikakogyo Co., Ltd., Tokyo). In experiments using cells isolated from the first leaves of seedlings, the number was counted without enzymic maceration because cell clusters were not formed. When the leaves of adult plants were used as the initial material, the number of cells and tracheary elements was counted after the formed cell clusters were macerated in an enzyme solution containing 1% (w/v) Macerozyme R-10 (Kinki Yakult Co., Ltd., Nishinomiya), 0.3 M mannitol with shaking at 90 strokes/min for 30 min.

RESULTS AND DISCUSSION

Leaf Age, Culture Method, and Osmolarity. In experiments to test various factors favoring tracheary element formation, adult plants of *Z. elegans* having seven pairs of leaves were selected and the leaves were used as plant material. Tracheary elements first appeared 6 days after culture in cells isolated from the youngest leaves, but 10 days after culture in those from older leaves. Tracheary element differentiation occurred at higher frequencies in cultures rotated at 10 rpm (7.0%) than in cultures not agitated (4.1%) or in cultures shaken at 84 strokes/min (2.9%). In Kohlenbach's work, the osmotic pressure was not adjusted. However, the adjustment of the osmotic pressure was necessary in this work, because cells showed hardly any growth unless the osmotic pressure was adjusted 0.3 to 0.4 M with mannitol.

[1] Abbreviation: NAA: α-naphthaleneacetic acid.

Table I. *Composition of the Culture Medium*

Macroelements	mg/l (mM)	Microelements	mg/l
KNO_3	2020 (20)	$MnSO_4 \cdot 4H_2O$	25
NH_4Cl	54 (1)	H_3BO_3	10
$MgSO_4 \cdot 7H_2O$	247 (1)	$ZnSO_4 \cdot 7H_2O$	10
$CaCl_2 \cdot 2H_2O$	147 (1)	$Na_2MoO_4 \cdot 2H_2O$	0.25
KH_2PO_4	68 (0.5)	$CuSO_4 \cdot 5H_2O$	0.025
		Na_2EDTA	37
		$FeSO_4 \cdot 7H_2O$	28
Organic Growth Factors	**mg/l**	**Growth Substances**	**mg/l**
Glycine	2	NAA	0.1
myo-Inositol	100	BA	1.0
Nicotinic acid	5		
Pyridoxine HCl	0.5	Sucrose	10 g/l
Thiamin	0.5		
Folic acid	0.05	Mannitol	36.4 g/l (0.2 M)
Biotin	0.05	pH 5.5	

Table II. *Effect of Various Concentrations of NAA and BA on Tracheary Element Formation*

Isolated mesophyll cells of adult plants were cultured for 8 days in the medium, whose composition is shown in Table I except for the concentrations of NAA and BA.

Concentration	Tracheary Elements/Total Cells
mg/l	%
NAA[a]	
0	0
0.05	4.7 ± 0.6[b]
0.1	8.2 ± 0.4
0.5	4.5 ± 1.6
1.0	3.6 ± 0.7
2.0	2.5 ± 0.5
BA[c]	
0	0
0.1	6.3 ± 1.5
0.5	7.8 ± 2.6
1.0	8.7 ± 0.7
2.0	3.2 ± 1.1

[a] In the presence of 1 mg/l BA.
[b] Mean ± standard deviation (N = 3).
[c] In the presence of 0.1 mg/l NAA.

Growth Substances. The presence of NAA and BA was essential for tracheary element formation in a suspension culture of cells isolated from the mesophyll (Table II). The favoring concentration of NAA and BA was 0.1 mg/l and 1 mg/l, respectively. 2,4-D could replace NAA. It has been reported by other workers (11, 18) that both auxins and cytokinins are essential for tracheary element formation, and a high ratio of cytokinin concentration to auxin concentration in a culture medium is a factor favoring tracheary element formation in multicellular systems as well as in the single cell system as in the work reported here.

Nitrogen Sources. Phillips and Dodds (11) reported that the nitrogen concentration in a medium affected the differentiation of tracheary elements. The effects of potassium nitrate and ammonium chloride at various concentrations were examined in our system. When the concentration of ammonium chloride was varied in the presence of 20 mM potassium nitrate, the highest percentage of cells differentiated to tracheary elements at a low concentration of ammonium chloride (0–1 mM) (Table III). When the concentration of potassium nitrate was varied in the presence of 1 mM ammonium chloride, the highest percentage of cells differentiated to tracheary elements at a moderate concentration of potassium nitrate (20 mM). At lower concentrations of potassium nitrate, the percentages of cells which differentiated to tracheary elements were lower and with the complete omission of potassium nitrate no tracheary elements were produced in the presence of 1 mM ammonium chloride. These results are contrary to those of Phillips and Dodds (11), who reported in a culture of explants of Jerusalem artichoke tubers that the largest number of tracheary elements was produced with the complete omission of inorganic nitrogen.

Initial Cell Population Density. Table IV illustrates the percentages of tracheary elements formed in cultures of the isolated mesophyll cells at various initial cell population densities. Tracheary element formation occurred at high frequency at initial cell population densities in the range 0.4 to 3.3 × 10^5 cells/ml. Therefore, this range of initial cell population density was selected. At lower densities, tracheary element formation occurred at lower levels.

Comparison of the First Leaves of Seedlings with the Youngest Leaves of Adult Plants as Material. Since the percentages of tracheary elements formed were low and a long time was needed to obtain plant material, when the youngest leaves of adult plants were used as starting material, it was necessary to improve the system. When the cells isolated from the first leaves of seedlings were used, tracheary element formation occurred earlier and at higher percentages than when those from the youngest leaves of adult plants were used. Tracheary elements first appeared on the 6th day in the culture of cells isolated from the youngest leaves of adult plants and the maximum percentage of tracheary element formation was only 10% (Fig. 1A). When the first leaves of seedlings were used as plant material, tracheary elements were observed first on the 3rd day and the percentage of their formation reached approximately 30% (Fig. 1B). Therefore, the first leaves of seedlings were considered to be a better material in this system than the youngest leaves of adult plants.

As regards favoring concentrations of NAA and BA for tracheary element formation, the results obtained in the cells isolated

Table III. *Effect of Various Concentrations of Ammonium Chloride on Tracheary Element Formation*

Isolated mesophyll cells of adult plants were cultured for 8 days in the medium, whose composition is shown in Table I except for the concentrations of ammonium chloride.

NH_4Cl	Tracheary Elements/Total Cells
mM	%
0	13.0 ± 0.9[a]
1	13.6 ± 2.7
5	4.7 ± 0.2
10	3.1 ± 3.7
20	0.2 ± 0.3

[a] Mean ± standard deviation (N = 3).

Table IV. *Effect of Initial Cell Population Density on Tracheary Element Formation*

Isolated mesophyll cells of adult plants were cultured for 8 days in the medium, whose composition is shown in Table I.

Initial Cell Density	Tracheary Elements/Total Cells
10^{-5} *cells/ml*	%
0.10	1.6 ± 0.8[a]
0.21	3.7 ± 1.7
0.42	6.8 ± 0.8
0.83	8.4 ± 1.4
3.32	7.5 ± 0.1

[a] Mean ± standard deviation (N = 3).

from the first leaves of seedlings were similar to those in the cells isolated from the youngest leaves of adult plants.

Time Course of Tracheary Element Formation and Cell Division in a Culture of Single Cells Isolated from the First Leaves of Seedlings. As shown in Figure 1B, a sudden increase in tracheary element number occurred between days 2 and 3 and the number reached a plateau within 24 h, indicating that the formation of tracheary elements occurred fairly synchronously during this period of culture. The number increased again from day 6 of culture. Cell number began to increase 2 days after culture and a gradual increase was observed thereafter. The highest proportion of tracheary elements (about 30% as a percentage of total cells) was found 3 days after culture and the proportion decreased gradually thereafter because the increase in cell number was greater than that in tracheary element number after day 4 of culture.

Observation of Isolated Mesophyll Cells and Differentiated Tracheary Elements. Cells immediately after isolation from the first leaves of seedlings are shown in Figure 2A. Single mesophyll cells contained both palisade and spongy parenchyma cells. Cells were fairly homogeneous in size and approximately 50 μm in length and 25 μm in width. There were no tracheary elements in the isolated cell population. Figure 2B shows the cells 4 days after

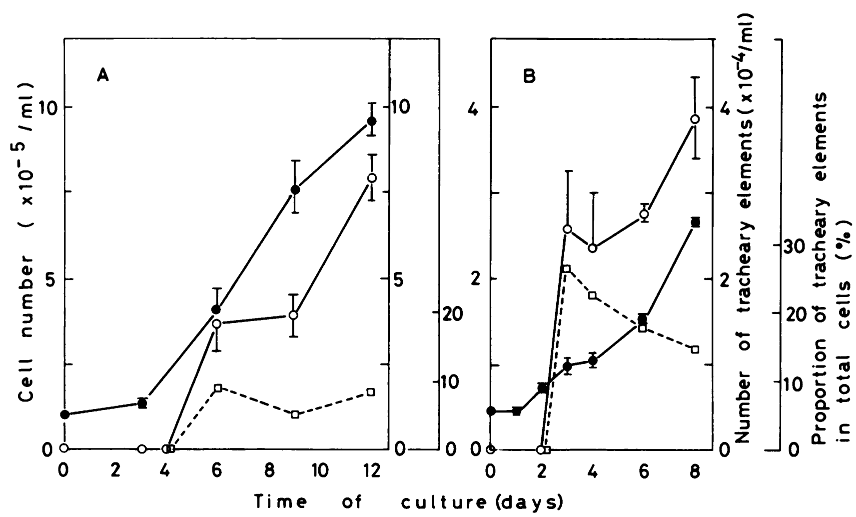

FIG. 1. Changes in number of tracheary elements (○), cell number (●), and proportion of formed tracheary elements as a percentage of total cells (□) during culture in suspension of single cells isolated from the mesophyll of *Z. elegans*. The youngest leaves of adult plants (A) and the first leaves of seedlings (B) were used as material.

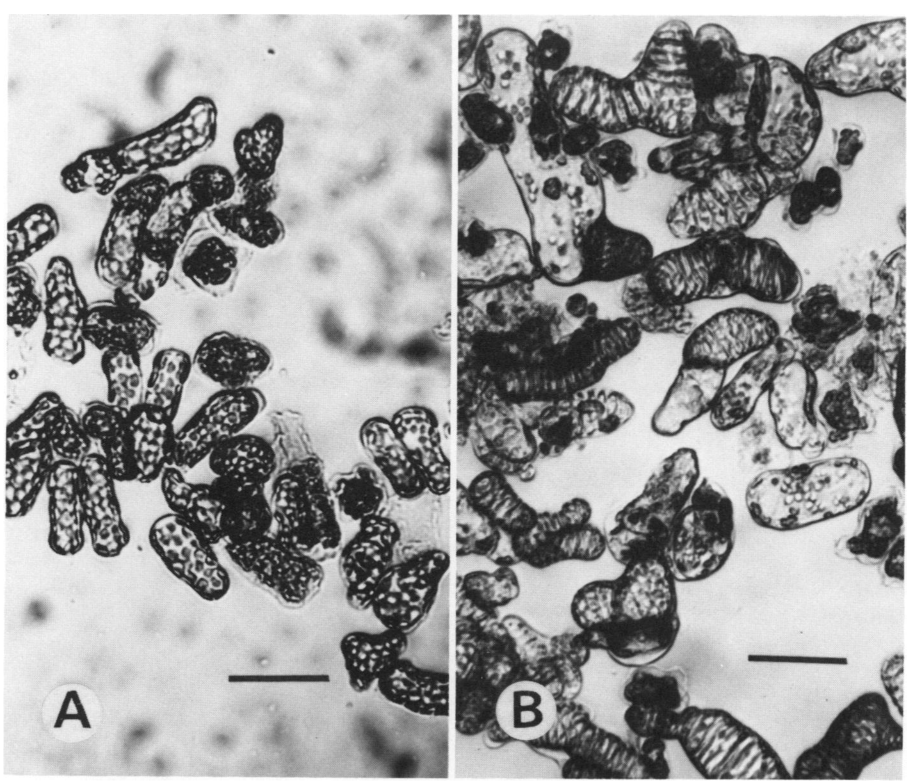

FIG. 2. Observation of isolated mesophyll cells. A: cells immediately after isolation from the first leaves of seedlings of *Z. elegans*, showing only parenchyma cells; B: cells after 4 days of culture, showing numerous tracheary elements.

culture. Some cells have differentiated to tracheary elements. The differentiated tracheary elements were 70 to 100 μm in length and 30 to 60 μm in width, indicating that the cells had expanded during culture.

Wound vessel member formation in segments of stems or roots (1, 13, 15) and tracheary element formation in slices of storage tissue (5, 8), in cultured callus (2, 18) and in a suspension cell culture (14) have been used as experimental systems for the study of cytodifferentiation to tracheary elements. The experimental system established in this work is considered to be useful for the study of tracheary element differentiation for the following reasons: (*a*) The initial cell population is composed of only single cells in this system and therefore, it is easy to determine whether individual cells which have differentiated have divided or not. (*b*) Each cell can receive equal stimulation to differentiate. (*c*) Isolated mesophyll cells differentiate to tracheary elements synchronously at least in the early stages of culture and the percentage of tracheary element formation reached approximately 30%, which is one of the highest rates that has ever been reported (5). (*d*) It is possible by using this system to follow visually the sequence of cytodifferentiation in individual cells.

For these reasons, this system of tracheary element formation from single cells isolated from the mesophyll of *Z. elegans* leaves is potentially a very efficient model system for physiological and biochemical studies to elucidate the mechanism of tracheary element formation and other studies on cytodifferentiation in higher plants.

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